Connexin Gene Transfer Preserves Conduction Velocity and Prevents Atrial Fibrillation

Running title: Igarashi et al.; Connexin gene therapy

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Abstract:

**Background** - Several lines of evidence have suggested that maintenance of atrial fibrillation (AF) depends on reentrant mechanisms. Maintenance of reentry necessitates a sufficiently short refractory period and/or delayed conduction, and AF has been associated with both alterations. Fibrosis, cellular dysfunction and gap junction protein alterations occur in AF and cause conduction delay. We performed this study to test the hypothesis that gap junction protein overexpression would improve conduction and prevent AF.

**Methods and Results** - Thirty Yorkshire swine were randomized into 2 groups (sinus rhythm (SR) and AF), and within each group into 3 subgroups: sham-operated control, gene therapy with adenovirus expressing connexin (Cx) 40 and Cx43 (n=5 per subgroup). All animals had epicardial gene painting; the AF group had burst atrial pacing. All animals underwent terminal study 7 days after gene transfer. SR animals had strong transgene expression but no atrial conduction changes. In AF animals, controls had reduced and lateralized Cx43 expression, and Cx43 gene transfer restored expression and cellular location to SR control levels. In the AF group, both Cx40 and Cx43 gene transfer improved conduction and reduced AF relative to controls.

**Conclusions** - Connexin gene therapy preserved atrial conduction and prevented AF.

**Key words:** atrial fibrillation; conduction; gene therapy; connexin
Atrial fibrillation (AF) is the most common cardiac arrhythmia in developed countries. In AF patients, maintenance of sinus rhythm (SR) correlates with improved survival, but current therapies for maintaining SR have limited efficacy and potential toxicities that often counteract this survival benefit. Overall, a safe and effective therapy for AF remains elusive. This deficiency has motivated us and others to develop novel interventions targeting the atrial substrate.

An important consideration when developing any therapy is the underlying disease mechanism. Several reviews have been written on this topic. Although some controversy exists about mechanism(s) for initiation of AF episodes, increasing levels of evidence support reentry as the dominant mechanism maintaining the arrhythmia after onset. Wavelength theory suggests that reentry could be disrupted by increasing either conduction velocity (CV) or tissue refractory properties. To date, manipulations to eliminate reentry in AF have focused largely on action potential prolongation, because tools to improve CV have been limited.

Improving myocardial CV requires either increasing tissue excitability or decreasing resistance to current flow. A potentially manipulatable component of tissue resistance is the level of intercellular connectivity, controlled predominately by gap junctions. The principle atrial gap junction proteins are connexins (Cx) 40 and 43. We hypothesized that atrial-specific Cx overexpression would increase CV, thereby preventing reentry and terminating AF. To address this hypothesis, we used a porcine model of burst pacing-induced AF and Cx gene delivery via a painting method. We have previously shown that epicardial gene painting causes homogeneous and complete transmural atrial gene transfer. With adenoviral vectors, effect onset occurred 2-3 days and peak effect occurred 7-10 days after gene transfer, making adenoviruses appropriate for proof-of-concept study. Here, we focus on gene therapy with...
Cx40 and Cx43 to evaluate effects on atrial CV and AF vulnerability.

**Methods:**

Please see online data supplement for full details of methods.

**Study protocol.** Thirty Yorkshire swine (20-30 kg) were randomized into 2 groups (sinus rhythm (SR) and AF groups), and within each group into 3 subgroups: sham-operated control, gene therapy with adenovirus expressing connexin (Cx) 40 and Cx43 (n=5 per subgroup).

During an initial procedure, animals underwent invasive electrophysiology study (EPS), gene painting with a solution containing 2g/L poloxamer-F127, 0.05g/L trypsin, and $1 \times 10^9$ pfu/ml of the indicated virus; and for AF animals, implantation of an atrial pacemaker. Burst atrial pacing was activated immediately after the procedure. On a daily basis, animals had clinical status assessment and 2-minute telemetry recording. On post-gene transfer day 7, animals underwent EPS and cardiac extraction for optical mapping, histology and molecular studies. All of these procedures were performed using standard methods, as previously reported. Cardiac rhythm was interpreted by two clinical cardiac electrophysiologists (TI, JKD). For subjective analyses (clinical assessment, rhythm analysis, histology and immunohistochemical studies), the investigators were blinded to specimen identity. For increased consistency, all histology and immunohistochemical analyses were performed in a single session.

**Statistical Analysis.** All data are presented as mean±SD. For rhythm analysis, probability of SR was assessed by Poisson regression using generalized estimating equations in order to address the animal-level clustering. In optical mapping, histology, immunohistochemistry and Western blot analyses, statistical differences were determined by related-measures ANOVA (post-hoc Tukey correction), which assumed that the outcome was correlated over time for each
animal. All statistical tests were conducted at the 0.05 significance level. Statistical analyses were performed using SAS9.2 GLM and GENMOD procedures.

Results:

Clinical observations and rhythm analysis. We assessed heart rhythm with daily 2-minute telemetry recordings. All animals in the SR group started in SR, remained in SR, and never had any observed rhythm other than SR on the daily recordings. In the AF group, we continued burst pacing with the 2-second on/off duty cycle through the telemetry recordings, and calculated %SR as the percent age of off-pacing segments showing SR per animal per day (Figure 1). In the AF group, we observed no rhythm other than AF or SR in any animal. In AF-controls, SR progressively declined over time. All control animals were in continuous AF 5.8±0.6 days after onset of burst pacing. The mean time to continuous AF for AF-controls in this study did not differ significantly from the time course we previously reported for control animals with this model.8,14,15

We analyzed probability of SR between AF animals using a generalized estimating equations model. We assumed that the probability of SR followed a Poisson distribution and working correlation matrix was first-order autoregressive. The results showed a significantly increased probability of SR for AF-AdCx40 (relative risk; 2.16, 95% confidence interval; 1.07 to 4.36, p<0.05) and AF-AdCx43 animals (relative risk; 3.69, 95% confidence interval; 1.97 to 6.93, p<0.01) compared to AF-controls. There was no statistically significant difference in SR% between AF-AdCx43 and AF-AdCx40.

We evaluated animal clinical status on a daily basis, and assessed echocardiograms at baseline and sacrifice (online supplement tables 1-2). Other than increased weight in the
SR-controls and SR-Cx43 animals relative to the other subgroups, there were no baseline differences between subgroups. The baseline weight difference in these SR subgroups had no apparent impact on the study as it did not correlate with measures of cardiac mechanical or electrical function. At sacrifice, echocardiography showed significantly increased left atrial size and decreased left atrial and left ventricular function in AF-controls relative to SR-controls. These structural alterations did not manifest in clinically apparent heart failure. Within the SR group, echo measurements did not differ between subgroups. In the AF group, left atrial size and function were improved in the Cx-treated animals relative to controls, consistent with the observed reduction in AF for those animals.

**Effects on Atrial Conduction.** We determined the effects of Cx gene transfer on atrial conduction using three methods: P-wave duration on 12-lead ECG, *in vivo* pacing at the SN and measurement of activation time at the tip of the LAA, and *ex vivo* optical mapping of an anterior left atrial tissue wedge. In the SR group, the P-wave duration did not differ between subgroups (Figure 2A). The P-wave duration was significantly longer in AF-controls compared to any other group. The P-wave duration in the AF-AdCx40 and AF-AdCx43 animals was significantly shorter than AF-controls, and not different than SR animals.

We observed similar behavior for both the *in vivo* atrial conduction time assessment (Figure 2B) and the *ex vivo* optical mapping (Figure 2C-D). In the SR group, intra-atrial conduction did not differ between subgroups. Intra-atrial conduction was significantly slower in AF-controls compared to all other groups, and conduction did not differ between AF-connexin subgroups and SR-controls. Interestingly, the atrial conduction data for AF control animals in this report are similar to those we previously reported in Amit et al., suggesting reproducibility across different animal groups over time with this model.8
Histological Evaluation. To assess for possible confounding of the conduction results from differences in structural remodeling between subgroups, we analyzed atrial histology (Figure 3). We examined previously reported histological parameters associated with AF: nuclear and cellular hypertrophy, interstitial inflammation, cellular myolysis and fibrosis. Nuclear enlargement, inflammation and myolysis were greater in AF-controls compared to SR-controls. Cellular hypertrophy and fibrosis did not differ between control groups. Within the AF and SR groups, histological measures did not differ significantly between subgroups. These data suggest that our observed differences in CV and AF prevention were a direct result of Cx gene transfer and not due to differences in remodeling.

Connexin Expression. We assessed Cx expression using Western blot analysis. We used an anti-Cx43 antibody that recognized total Cx43, and we distinguished the differentially phosphorylated variants by their molecular weights. Similar to other reports, we observed only a single Cx40 band, so we could not assess Cx40 phosphorylation.16

In the SR group, the expression of total Cx40 was greater in SR-AdCx40 animals (Figure 4A-B) and the expression of both total and phosphorylated Cx43 were greater in SR-AdCx43 animals (Figure 4C-D) compared to SR controls.

Comparing AF to SR controls, expression of total Cx40 was not significantly different (Figure 4A-B), but expression levels of total and phosphorylated Cx43 were markedly diminished in the AF-controls (Figure 4C-D). We saw significantly increased Cx43 expression in the AF-AdCx43 animals, and significantly increased Cx40 in the AF-AdCx40 animals relative to AF-controls. The levels of both total and phosphorylated Cx43 expression in the AF-AdCx43 animals were similar to the levels found in SR-controls.

Connexin Localization. We evaluated the percentage of expressed connexin located at the
intercalated disk using immunohistochemistry and confocal imaging (Figure 5A). In the SR group, the relative percentage of connexin at the intercalated disk did not change with Cx43 overexpression. There was a slight but significant decrease in the relative percentage of intercalated disk Cx40 for the SR-Cx40 group (Figure 5B-C). In both the AF-control and AF-Cx40 subgroups, we saw an increase in the relative percentage of lateralized Cx43 compared to SR animals. AdCx43 gene therapy restored intercalated disk localization of Cx43 to the same level observed in SR group animals. There were no differences in Cx40 lateralization between AF subgroups.

**Cross-regulation of Cx40 and Cx43.** We used Western blot analysis to examine for changes in Cx40 with AdCx43 exposure or changes in Cx43 with AdCx40 exposure. We confirmed that the antibodies were sensitive and specific to the indicated Cx proteins (online supplement figure). In the SR group, animals receiving AdCx40 had no significant difference in total or phosphorylated Cx43 expression compared to SR-controls (Figure 6A-B). However, the SR-AdCx43 animals had significantly increased Cx40 expression compared to SR-controls (Figure 6C-D). In the AF group, we saw no difference in Cx43 for the AF-AdCx40 animals or in Cx40 for the AF-AdCx43 animals relative to AF-controls. Thus, AdCx40 had no effect on Cx43 levels in either SR or AF. AdCx43 increased Cx40 expression during SR, but this effect was lost with atrial burst pacing.

**Toxicity evaluation.** The possibility of proarrhythmia is a principle concern when manipulating cardiac electrophysiology. We evaluated for atrial or ventricular proarrhythmia or signs of other toxicity during the in-life phase by assessing daily clinical measures and telemetry for atrial or ventricular arrhythmias, and at termination study by assessing 12-lead ECG parameters, echocardiography, tachycardia inducibility, MAPD$_{90}$, and by looking for early
afterdepolarizations (online supplement table 2). We saw no evidence for toxicity in any animal.

Discussion:

We found in an AF cohort that atrial gene transfer of either Cx40 or Cx43 successfully preserved atrial conduction and prevented development of persistent AF. Interestingly, in the SR group, no phenotype emerged in spite of successful transgene expression. To achieve these results, we used epicardial gene painting, which we and others have validated as a method for homogeneous, complete transmural gene transfer to atrial areas accessible from the epicardium (anterior free walls and appendages, posterior area between the pulmonary veins, not the atrial septum or the posterior atria between the vena cavae and the right pulmonary veins).12 We tested animals 7 days after gene transfer, at the time that we have previously shown to be peak effect with adenoviral vectors delivered using this method.8 Our goal with this study design was to eliminate potential confounders such as unstable, heterogeneous, or waning gene expression.

Like all research, our model and data have important limitations that need to be considered when interpreting the data. To every extent possible, we prospectively compensated for these limitations with our study design. We simultaneously performed the gene transfer and pacemaker implantation because we knew from prior experience that heart failure prevents the animals from tolerating a later open-chest procedure. We could only assess rhythm manually during limited portions of each day because available telemetry devices cannot distinguish burst pacing from AF. Limited patience of the pigs prevented longer-duration recordings. Similar to our previous report,8 in this study we continued 2 second on/off burst pacing during telemetry
recording to obtain the most rigorous possible assessment of resistance to fibrillation (i.e. discontinuing the burst pacing would allow recording of a single AF termination event; continued burst pacing allows observation of re-induction and repeated termination events per recording). Limitations in ability to measure conduction velocity across the entire atrial surface motivated our use of 3 complementary methods, all of which showed general agreement although with some variability in effect magnitude. Our study was prospectively designed and powered to distinguish differences in CV and AF vulnerability with Cx gene transfer. As such, we cannot definitively exclude the possibility of false negative results for some of the measures showing no statistically significant difference (e.g. efficacy differences between AF-Cx40 and AF-Cx43 subgroups, or differences in Cx expression and CV between AF-connexin and SR-control groups). Further investigation in a study prospectively designed and appropriately powered to assess these issues could supplement our current data.

**AF/CHF model.** To put the current findings into context, it is important to briefly review our previous observations with this model, and to compare the porcine AF/CHF model to the available data from humans and from other experimental models. The dominant features of our model are progression to sustained AF with continued burst pacing over several days and aggressive development of heart failure from the uncontrolled ventricular rate. In prior study, we found four-chamber dilation and LVEF of 28% 3 weeks after initiation of burst pacing, and LVEF 22% at 5 weeks. In the current study, we found evidence of left atrial dilation, and slightly reduced LVEF (53%) without change in LV diameter after 1 week of burst pacing. Evaluation of atrial histology in previous study showed severe myolysis, inflammation, cellular and nuclear hypertrophy and only modest fibrosis at the 3- and 5-week timepoints. In our current data, we saw the beginnings of myolytic, inflammatory, and hypertrophic responses after
1 week, but we saw no difference in fibrosis between AF and SR controls. EP study from a previous cohort revealed conduction velocity slowing (P-wave duration baseline 54 ms, 1 week 90 ms, 3 weeks 100 ms; SN to LAA conduction time baseline 72 ms increasing to 90 ms at 1 week) in a similar range as that reported in our current study.\textsuperscript{8}

Histological and conduction data for human AF are limited. The majority of available data come from measurements on tissue samples taken during cardiac surgery, and comparisons are generally made between SR and AF patients with valve disease or coronary atherosclerosis. Data on patients with any significant degree of LV dysfunction are extremely limited. General trends across the human studies include evidence of atrial fibrosis, inflammation, myolysis, and reduced intra-atrial conduction velocity.\textsuperscript{17,18}

Animal models potentially supplement the limited human information. It is important to keep in mind when reviewing the animal data, however, that each model reflects a different facet of the human disease, but none of these models (including ours) provides a complete picture of the diverse set of conditions that make up human AF.

The goat atrial burst pacing model is remarkable for sustained AF in the absence of structural ventricular disease, an important distinction between the goat and pig models. The goat model likely emulates lone AF in humans. In a series of investigations, Allessie and colleagues reported extensive atrial myolysis and inflammation but no atrial fibrosis or CV slowing even after months of AF.\textsuperscript{19-21}

Several canine models have been reported, each with distinct features. A sterile pericarditis model, potentially analogous to post-operative AF, has CV slowing and inflammation.\textsuperscript{22} An atrial tachypacing model simulates sustained atrial tachycardia, with increased AF inducibility but without sustained AF. In one study, CV decreased approximately
25% over several weeks, but half of that change occurred within the first 24 hours of atrial tachypacing. Another study reported a 50% increase in P-wave duration, consistent with significant CV slowing, after 2-6 weeks of burst pacing. A ventricular tachypacing model with increased AF inducibility but without sustained AF provides the only data for atrial remodeling in the setting of impaired LV function. Key features of that model included atrial fibrosis and either heterogeneous or reduced atrial CV.

Overall, then, the relevant features of our model include rapid progression to persistent AF over several days, steady progression of severe atrial myocyte pathology and ventricular failure over a few weeks, and reduction in atrial conduction velocity prominently in the first week with continued decline over at least a 3-week period. Similarities between our model and the available human data include decreased atrial conduction velocity and prominent myocyte pathology. Important differences include limited fibrosis and aggressive onset of heart failure in our model. Relative to the other animal models, direct comparisons are difficult because no other model has the combined features of persistent AF and heart failure. The goat model has persistent AF, but without heart failure. The canine ventricular tachypacing model has prominent ventricular failure, but without sustained AF. As such, our model likely has some features driven by interaction between persistent AF and heart failure that are not captured by other animal models.

Connexins and AF. Loss-of-function mutations in both Cx40 and Cx43 have been associated with lone AF, suggesting a role for cellular coupling in vulnerability of structurally normal hearts to AF. Connexin data are inconsistent across studies of diseased human hearts. Kostin et al. found decreased and lateralized Cx43 expression and heterogeneous Cx40 expression (increased in the right atrial free wall, decreased in the right atrial appendage) without overall
Rucker-Martin et al. found reduced expression of phosphorylated Cx43 and total CX40, with lateralization of Cx43.28 Nao et al. and Gaborit et al. saw no significant change in Cx43 levels and decreased Cx40, and Wetzel et al. found increased levels of both connexins.29-31 The heterogeneous expression observed by Kostin suggests that biopsy site may account for the differing Cx40 expression levels observed in the other reports. No explanation is available for the variability in Cx43 data. Overall, in spite of the variability in these reports, consistent findings included lateralization and dephosphorylation of Cx43 (where assessed) suggesting that the level of functional Cx43 is diminished in human AF.

In the goat atrial burst pacing model, Van der Velden et al. saw no change in overall expression levels of Cx40 or Cx43, but they noted Cx40 expression heterogeneity similar to Kostin, dephosphorylation of Cx43, and lateralization of both connexins.20,21 In the canine sterile pericarditis model, Cx43 expression was diminished throughout the myocardium, and essentially undetectable in the epicardial layer.22 In the canine atrial tachypacing model, Cx43 mRNA expression was reduced approximately 67% in one study, but another study found a 2-fold increase in Cx43 protein level with a comment that Cx43 was more prominent at the lateral cell borders.32,33 In the canine ventricular tachypacing model, both dephosphorylation and lateralization of Cx43 were uniformly observed.16,24,25 Cx43 levels were variably reported to be either unchanged or increased, and Cx40 levels were unchanged compared to SR controls.16,33 Bikou et al. reported a decrease in Cx43 expression in a model of porcine AF similar to ours.34 Thus, the animal data are similar to the human studies in that Cx expression levels varied by study but Cx43 lateralization and dephosphorylation were consistent findings across multiple reports in different animal models.

Reports of peptides that modulate connexin activity have shown inconsistent effects in
canine models of acutely induced AF. Shiroshita-Takeshita et al. found that rotigaptide had no effect on normal canine atria, slight but statistically significant improvement in CV for atrial or ventricular tachypacing animals, and considerable improvement in CV when administered to dog atria prior to ischemic insult. The duration of self-limited, acutely induced AF was shortened by rotigaptide only in the ischemic model. In contrast, Guerra et al. found that rotigaptide significantly improved conduction velocity in normal dogs and dogs with chronic mitral regurgitation but not ventricular tachypacing dogs. The mitral regurgitation dogs also had significant reduction in the duration of acutely induced AF episodes. Discrepancies between these results (e.g. improved CV in normals for one study and not the other) are difficult to explain based on the methods used, since they are similar between the studies. Relevance of the rotigaptide data to our results are difficult to assess given these discrepancies in canine results, and the unclear comparability of acute AF episode duration to the persistent AF of the porcine model. The drug mechanism for connexin-modulating peptides is incompletely understood, but it seems to require receptor-binding, protein kinase C activation and modulation of connexin phosphorylation state (see review by Dhein et al. for a more comprehensive discussion of drug mechanism). Any of these steps could be impaired in the canine models, thus limiting drug efficacy. Connexin expression levels are another important parameter to consider for the canine studies of rotigaptide. If the protein is not there, then the drug is unlikely to affect its function. Basic pharmacokinetics also need to be considered when assessing drug effect. Is the drug getting to the target at the time and in the concentration necessary to achieve its effect? None of these issues is investigated in the rotigaptide studies.

In our AF-heart failure model, decreased Cx43 expression occurred in the setting of CV slowing and onset of persistent AF. Gene transfer-induced increase in either Cx40 or Cx43
prevented the CV decrease and stopped the fibrillation, suggesting a mechanistic link between connexin expression, CV and AF in this model, a finding supported by recent data from Bikou et al. In our study, the AF-controls had left atrial dilation and reduced ejection fraction relative to AF-Cx40 and AF-Cx43 animals, likely a result of differences in AF burden. These gross structural changes did not manifest in differences at a microstructural or cellular level. Atrial fibrosis and cellular myopathic changes were similar between groups as was left ventricular structure and function, so those potential confounders could not explain the observed result. Since Cx43 was decreased but Cx40 was not, our data suggest that Cx40 and Cx43 may be interchangeable in their ability to preserve cellular connectivity and conduction.

**Absence of a phenotype in SR animals.** An interesting observation in this report is the absence of any discernable increase in CV for the SR animals paired with the CV preserving effects in the AF animals. These data verify a role for connexins in determining CV but suggest that other obstacles to conduction, unrelated to cellular connectivity, are the principle determinants of CV under normal circumstances.

Our data are the first report of atrial CV after Cx 40 and 43 overexpression. The prior literature focused primarily on decreasing or eliminating either Cx40 or Cx43, and the resulting effects on atrial CV have been inconsistent. Thomas et al. found no difference for *in vivo* measures of atrial CV when comparing wild type to heterozygous Cx43-deleted mice. In contrast, Beauchamp et al. observed a 44% reduction in CV with *in vitro* measurement of atrial myocytes from mice homozygous for Cx43 deletion. Beauchamp et al. also observed an increase in CV *in vitro* with atrial myocytes isolated from Cx40 knock-out mice. Leaf et al. saw regional heterogeneity but overall no change in atrial CV with *ex vivo* optical mapping of either homozygous or heterozygous Cx40 knock-out mouse hearts, and Verheule et al. found a
30% decrease in atrial CV *in vivo* with heterogeneous Cx40 deleted mice.\textsuperscript{41} In a study inducing limited Cx43 overexpression, Bikou et al. found increased CV in right but not left atria.\textsuperscript{34}

Methodological differences likely account for some variability in this literature. The *in vivo* methods used by Thomas and Verheule are the least precise or controllable but arguably the most relevant. The *ex vivo* mapping of Leaf has the advantage of better resolution and therefore potentially more reliable CV measurement, but these data need to be considered within the context of the metabolic changes resulting from loss of blood perfusion, the use of voltage-sensitive dyes and excitation-contraction uncouplers, and the potential tissue damage from harvest and manipulation. Beauchamp’s *in vitro* mapping has the greatest likely precision and control, but the extreme manipulations necessary to create this model limit application to the *in vivo* environment. To overcome these limitations, we evaluated atrial conduction with a combination of *in vivo* and *ex vivo* methods. We saw similar trends in the overall effect across methods (no change in SR, decrease in AF, preservation to SR levels in AF with Cx gene transfer), but the magnitude of conduction effects differed with method and conditions.

Another potential confounder is compensatory change in expression of untargeted connexins. In particular, Beauchamp’s observations could potentially be explained by the level of Cx43 expression alone, since Cx43 increased in the Cx40 knock-out mice where CV increased, and decreased in the Cx43 knock-out along with decreased CV. Thomas and Leaf saw no compensatory changes and no change in overall CV. We saw an increase in Cx40 expression for the SR-Cx43 group, but no compensatory change in any other group. The potential interaction between Cx40 and Cx43 was explored by Cottrell et al. who evaluated various combinations of Cx40 and/or Cx43 expressing cells *in vitro*.\textsuperscript{42,43} They found a shift to a lower conductance state when mixed Cx40/43 channels docked with pure Cx43 channels but not
when the mixed channels docked with pure Cx40 channels. These results suggest a more complex relationship than can be estimated by looking only at Cx40:Cx43. Further study is warranted to increase our understanding of this issue, but that is beyond the scope of the current report.

In our data, the absence of a phenotype in the SR group does not appear to be connected to Cx localization, phosphorylation or function. We saw no change in the relative amount of Cx43 at the intercalated disk for the SR-Cx43 subgroup, and only a subtle alteration in Cx40 localization for the SR-Cx40 subgroup. We did not see any change in the ratio of phosphorylated to total Cx43 with gene transfer. Successful preservation of conduction in the relevant AF groups further suggested appropriate processing and function of the transgenic connexins. The SR result suggests that under normal operational circumstances, gap junctional resistance is not the principle limitation to conduction, and that increased expression beyond the normal level cannot overcome other determinants of conduction velocity.

**Conclusion.** In this study, we have shown that atrial overexpression of Cx40 and Cx43 had no discernable effect on conduction in SR animals, but both transgenes preserved CV and prevented sustained AF. We also found considerable evidence for safety of this approach. For these proof-of-principle data, we focused on the short-term connexin gene transfer to assess conduction velocity and anti-fibrillatory effects. Future study with permanently expressing vectors like AAV or lentivirus would allow evaluation over the longer-term to assess interaction with varying levels of atrial pathology. Our results show that atrial-specific gene therapy with gap junction protein provides a novel paradigm for AF treatment based on improvement of intercellular communication.
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Conflict of Interest Disclosures: Dr Donahue has an ownership interest in Excigen Inc.

References:


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Figure Legends:

**Figure 1.** Telemetry recordings.  **A.** Representative ECG tracings in the AF group at day 7. Sinus rhythm percentage (SR%) was calculated using the formula.  **B.** Summary data showing SR% for all animals in the AF group over time since gene therapy.  See text for statistical analysis.

**Figure 2.** Conduction assessment.  **A.** P-wave duration measured during SR 7 days after gene transfer.  **B.** Intra-atrial conduction time measured while pacing the sinus node and recording at the tip of the left atrial appendage 7 days after gene transfer.  **C.** Representative isochronal maps of left atrial epicardial activation time from optical mapping.  Pacing site is marked.  **D.** Summary data showing longitudinal and transverse conduction velocities from optical mapping.  **p**<0.01 AF- vs. SR-controls; † **p**<0.05, †† **p**<0.01 between AF subgroups.

**Figure 3.** Atrial Histology.  Representative images of atrial tissues stained with Hematoxylin and eosin (A) and Masson’s trichrome (B).  **C.** Comparison of histological changes based on a previously reported, standardized scale.14  * **p**<0.05, ** **p**<0.01 AF- vs. SR-controls; p=NS within AF and SR groups.  **D.** Quantification of fibrosis from Masson’s trichrome-stained sections.  p=NS among all animals.

**Figure 4.** Transgene expression.  **A.** Representative immunoblots of Cx40 and GAPDH from control and AdCx40 animals.  **B.** Quantification of band intensity for Cx40, normalized to GAPDH expression.  **C.** Representative immunoblots of Cx43 and GAPDH from control and
AdCx43 animals. P1 and P2 indicate phosphorylated Cx43 bands, and NP indicates non-phosphorylated Cx43.  

**D.** Quantification of band intensity for Cx43, normalized to GAPDH expression.  *p<0.05, **p<0.01 AF- vs. SR-controls; † p<0.05, †† p<0.01 for subgroup comparisons within AF- or SR-groups.**

**Figure 5.** Connexin localization.  

**A.** Illustration of procedure to quantify connexin localization. Connexin location was determined using immunohistochemistry and confocal imaging. Only longitudinal sections of myocytes were evaluated. An investigator blinded to study group identified intercalated disk (white dotted boxes) of all myocytes in a high power field. We used image quantification software to quantify signal intensity and then calculated the ratio of intercalated disk connexin to total.  

**B.** Representative immunohistochemistry of Cx40 (yellow) and Cx43 (green) in the SR and AF groups.  

**C.** Quantification of signal intensity showing intact localization of Cx40 and Cx43 to the intercalated disk after gene transfer.  **p<0.01 AF- vs. SR-controls. †p<0.05 within SR subgroups, ††p<0.01 within AF subgroups.**

**Figure 6.** Cross-regulation of non-targeted connexins.  

**A.** Representative immunoblots of Cx43 and GAPDH from control and AdCx40 animals.  

**B.** Quantification of band intensity for Cx43 normalized to GAPDH expression in AdCx40 and control animals.  

**C.** Representative immunoblots of Cx40 and GAPDH from control and AdCx43 animals.  

**D.** Quantification of band intensity for Cx40 normalized to GAPDH in AdCx43 and controls animals.  **p<0.01 vs. SR-controls.**
SR% = \frac{\text{number of SR non-pacing segments}}{\text{total non-pacing segments}} \times 100\%
IC-disk (%) = \frac{\text{IC-disk intensity}}{\text{Total intensity}} \times 100\%
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Data Supplement
### Online supplement table 1: clinical observations

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</table>

Legend: all observations are based on a 0 to 1 scale where 1 is completely normal and 0 is complete absence of activity. Observations are made by animal care staff who are blinded to patient group. * p = 0.005 by ANOVA, Tukey's post-hoc test showed significant difference in SR-control and SR-Cx43 vs. other subgroups.
### Online Supplement Table 2: Electrocardiography, echocardiography and electrophysiology study results

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<tr>
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<td>71 ± 4</td>
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<tr>
<td>PR interval (ms)</td>
<td>102 ± 12</td>
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<td>QRS duration (ms)</td>
<td>70 ± 15</td>
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<td>QTc interval (ms)</td>
<td>415 ± 14</td>
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<td>RR interval (ms)</td>
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<td>LA diastolic diameter (cm)</td>
<td>2.4 ± 0.3</td>
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<td>LV diastolic diameter (cm)</td>
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<td>LAEF (%)</td>
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<td>LV EF (%)</td>
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<td>average RA MAPD90 (ms)</td>
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<td>average LA MAPD90 (ms)</td>
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<td>basal RV MAPD90 (ms)</td>
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<td>basal LV MAPD90 (ms)</td>
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<tr>
<td>RA ERP (ms)</td>
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<td>LA ERP (ms)</td>
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<td>73 ± 4</td>
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<td>PR interval (ms)</td>
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<td>QRS duration (ms)</td>
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<td>QTc interval (ms)</td>
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<td>RR interval (ms)</td>
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<tr>
<td>LA diastolic diameter (cm)</td>
<td>2.5 ± 0.3</td>
<td>2.4 ± 0.3</td>
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<tr>
<td>LV diastolic diameter (cm)</td>
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<tr>
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<td>average RA MAPD90 (ms)</td>
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<td>average LA MAPD90 (ms)</td>
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<td>basal LV MAPD90 (ms)</td>
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<td>RA ERP (ms)</td>
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<td>LA ERP (ms)</td>
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<td>RV ERP (ms)</td>
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<td>250 ± 26</td>
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<tr>
<td>LV ERP (ms)</td>
<td>196 ± 13</td>
<td>226 ± 15</td>
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Legend: SR sinus rhythm, AF atrial fibrillation, QTc Bazett’s corrected QT interval, RA right atrium, LA left atrium, RV right ventricle, LV left ventricle, EF ejection fraction, MAPD90 monophasic action potential duration to 90% repolarization, ERP effective refractory period measured at drive cycle length 400 ms, *p<0.05, **p<0.01 AF- vs. SR-controls, †p<0.05, ††p<0.01 AF subgroups vs. control ANOVA. At baseline, there were no statistically significant differences between subgroups. At termination study, SR subgroups were not statistically different from each other.
Online Supplement Figure: Antibody specificity. To confirm that the Cx43 antibody did not cross-react with Cx40, and that the Cx40 antibody did not cross-react with Cx43, and that the respective antibodies reacted to both the transgene and native pig connexins, we infected Cos-7 cells with adenoviruses encoding pig Cx40 and Cx43 in addition to the adenoviruses used in the study animals encoding human Cx40 and rat Cx43. After 2 days, we harvested cells, isolated proteins and performed Western blot analysis using methods described below. The results show that the Cx40 antibody does indeed react with both native pig and transgenic human connexin 40, and the Cx43 antibody reacts with native pig and transgenic rat Cx43. The Cx40 antibody does not react with Cx43, and the Cx43 antibody does not react with Cx40. Above and immediately below the Cx40 band, two false bands are apparent in all lanes, including the negative control (uninfected Cos07 cells). Likewise, a false band is noted in the negative control lane and very faintly in the Cx40 lanes for the Cx 43 antibody. These bands were excluded from the area of intensity analysis.
Online Supplement Methods:

Adenovirus vectors: The recombinant adenoviruses used in this study were first-generation adenoviruses, rendered replication incompetent by deletion of the E1 and E3 genes. Human Cx40 and rat Cx43 genes were obtained from the American Type Culture Collection (ATCC, Manassas, VA), cloned into a shuttle plasmid within a construct containing the CMV immediate-early promoter/enhancer—gene of interest—internal ribosome entry site—enhanced green fluorescent protein. The shuttle plasmid was co-transfected with a second plasmid containing the remaining elements of the adenoviral genome into Cre-expressing HEK-293 cells, using the method originally reported by Hardy et al. The resulting viral lysate was plaque purified and then expanded into sequentially larger tissue culture flasks from a single T25 flask up to multiple T175 flasks to give sufficient material for quality analysis.

Further expansion of virus stock was performed using conventional cell culture, and virus purification was performed using Adenopur columns following the manufacturer’s instructions (Puresyn, King of Prussia, PA). Virus particle concentration was calculated from the absorbance at 260 nm and the ratio of absorbance at 260 and 280 nm (A260/A280). The infectious titer was determined by plaque assay. Transgene expression was confirmed by Western blot analysis from the products of infection in Cos-7 cells, a non-permissive cell line. The absence of replication competent virus was confirmed by PCR with primers directed to
the adenoviral E1 gene, which is deleted from the recombinant viruses but present in the wild-type virus. The resulting virus stock was stored at -80°C until use. Virus storage solution consisted of phosphate-buffered saline containing 1 mM MgCl₂ and 10% glycerol.

**Painting solution:** The painting solution was made as previously reported,² by chilling Krebs’ solution to 4°C and adding poloxamer F127 (BASF, Mt. Olive, NJ) to achieve a stock concentration of 2.5g/L. The resulting mixture was left stirring at 4°C overnight to fully dissolve the poloxamer. Prior to starting the procedure, cold adenovirus and trypsin stock solutions were added to the poloxamer/Krebs’ solution. The fully composed painting solution consisted of 2g/L poloxamer F127 (BASF, Mt. Olive, NJ), 0.05g/L trypsin (250 USP units/mg, BD Diagnostics, Sparks, MD), and 1x10⁹ pfu/ml of the indicated virus in Krebs’ buffer. Immediately before use, the painting solution was warmed in a 37°C water bath to achieve a firm gel consistency.

**Animal studies:** Thirty Yorkshire swine were randomized into 2 groups (sinus rhythm (SR) and AF groups), and within each group into 3 subgroups: sham-operated control, gene therapy with adenovirus expressing connexin (Cx) 40 and Cx43 (n=5 per subgroup). Please refer to online supplement table 1 for baseline data on the animals included in the study.

The animals for this study were maintained in accordance with the Policy on Humane Care and Use of Laboratory Animals from the Office of Laboratory Animal Welfare, National Institutes of Health. The experimental protocol was approved by the Institutional Animal
Care and Use Committee.

Our study was designed to test the primary hypothesis that Cx gene transfer would increase CV and prevent AF. As such, our sample size calculations were based on the most applicable information that we could find in the literature. From those data, we designed our study to detect CV changes of 15% with variance of 5 and to detect at least a 30% risk reduction for AF, so subgroups of 5 animals each would give approximately 90% power at the 0.05 level of significance. Randomization was performed using the random number generator function of Microsoft Excel, and animals were assigned to both group and subgroup in a single randomization procedure.

During an initial procedure, animals underwent invasive electrophysiology study (EPS), gene painting, and for AF animals, implantation of an atrial pacemaker. Immediately after the procedure, burst atrial pacing was activated. The burst protocol paced at 42 Hz frequency, 7.5 V output for 2 second increments alternating with 2 second period of no pacing. On a daily basis, animals had clinical status assessment and recording of a 2 minute telemetry strip. On post-gene transfer day 7, animals underwent terminal EPS and cardiac extraction for optical mapping, histology and molecular studies.

**Electrophysiology study (EPS), gene transfer and pacemaker implantation:** EPS was performed as previously reported. All recordings were taken during sinus rhythm or constant rate pacing at a 400 ms cycle length. For the initial study, all animals were in sinus
rhythm. For the termination study, we cardioverted animals in AF at least 30 minutes before EPS. We recorded a 12-lead ECG with standard lead positions.

Animals were sedated with telazol 1.5 mg/kg IM, xylazine 1.5 mg/kg IM, and ketamine 1.5 mg/kg IM. After animals were unresponsive, we intubated and ventilated the animals, maintaining anesthesia with isoflurane 0.5-1.5%. We scrubbed the chest for 5 minutes each with chlorhexidine and betadine scrub, sprayed the chest with betadine solution, draped the surgical area with sterile towels and sheets, and opened the chest by median sternotomy. The pericardium was incised and EPS was performed. We acquired monophasic action potential (MAP) recordings using a 7-French MAP catheter (Boston Scientific, Natick, MA) positioned sequentially at the center of 10 pre-defined epicardial atrial regions and in the basal region of the ventricles, adjacent to atrial sites 4 and 9 as previously described. MAP recordings were continued through the post-burst pacing pause to evaluate the post-pause beat for early afterdepolarizations (EADs). The MAP duration was measured as the interval from the steepest part of the MAP upstroke to the level of 90% repolarization (MAPD90) during regular pacing with a drive train cycle length of 400 msec.

After MAP recordings, bipolar electrodes were placed at the sinus node and left atrial appendage. Electrogram recordings were made during sinus node pacing at 400 ms drive train. The distance between catheters was measured and recorded. Catheters were repositioned to area 4 of the right atrium, area 9 of the left atrium, and to the basal left and
right ventricles immediately adjacent to the atria. Effective refractory period was measured for each chamber by delivering an 8 pulse drive train at 400 ms coupled to a premature stimulus. The coupling interval of the premature stimulus was decreased in 10 ms increments, and the ERP was defined as the longest coupling interval that fails to conduct to the local tissue on the quadripolar catheter. After ERP measurement, burst atrial pacing of left and right atria was performed at maximum output, 60 ms coupling interval for 10 and 30 seconds. Any atrial arrhythmias induced by either single extrastimulus or burst pacing were documented and recorded.

After EPS, we performed the gene painting procedure as previously described. The virus/trypsin/poloxamer gel was painted onto the atria using a rounded bristle, flat paintbrush composed of camel hair. The heart was manipulated to expose all epicardial surfaces, and all portions of the atria reachable by an epicardial approach were painted (including the anterior and lateral portions of each atria from the vena cavae on the right to the left pulmonary veins on the left, the posterior-inferior atria around the coronary sinus, and the posterior recess between the pulmonary veins). Each painting area was coated twice for 60 seconds each, and approximately 5 minutes were given between painting coats to allow absorption. After painting, the heart was left exposed to air for 10 minutes to allow virus penetration. After painting was complete, the sternum and overlying fascia and skin were closed. During closure a mediastinal tube was placed. When the chest was closed, all air was evacuated
through the tube and then the tube was pulled.

We implanted a single chamber pacemaker using conventional techniques, placing an active-fixation lead through the right external jugular vein into the right atrium (RA) connecting to a pacemaker in a subcutaneous pocket in the neck. At the end of the case, we activated the pacemaker to burst pace the RA at 42 Hz frequency and 7.5 V output for 2 second increments alternating with 2 second pauses between burst episodes.

**Rhythm Analysis:** We recorded a 2-minute ECG strip with a 6-lead telemetry system daily for rhythm analysis as previously reported. Animals were awake and alert at consistent levels from one reading to the next. Two investigators (TI and JKD) blinded to study group assessed rhythm during the 2-second off-pacing segments. We prospectively defined SR as the presence of any sinus beats during the off-pacing segments and AF as the absence of organized sinus beats, with an erratic baseline and irregularly, irregular ventricular response. No other rhythms were seen in this study.

**Optical Mapping:** After extraction, the heart was immediately dunked in iced-cold cardioplegic solution (135mM NaCl, 30.0mM KCl, 1.80mM CaCl₂, 10.0mM HEPES, 0.50mM MgSO₄, 0.90mM NaH₂PO₄, and 5.5mM dextrose, pH=7.5). The left circumflex coronary artery and the LA were dissected free of the ventricles and the artery was cannulated. The severed ventricular branches of these vessels were ligated and the LA was perfused with oxygenated Tyrode’s solution (135mM NaCl, 4.0mM KCl, 1.80mM CaCl₂, 10.0mM HEPES,
0.50mM MgSO₄, 0.90mM NaH₂PO₄, and 5.5mM dextrose, pH=7.5) to maintain a perfusion pressure at 50-60 mmHg. The tissue was immersed in temperature-controlled (36±1°C) perfusate and stabilized against a flat imaging window by application of a gentle constant pressure via a movable piston. For consistency, the section of tissue corresponding to Kikuchi area 9 was mapped in all animals (anterior inferior left atrium adjacent to the left atrial appendage, see Kikuchi et al. for diagram).² The preparation was perfused with the excitation-contraction uncoupler blebbistatin (Sigma, St. Louis, MO, final concentration: 1 μM) for 15 minutes and then stained with the voltage sensitive dye di-4-ANEPPS (Sigma, St. Louis, MO) by perfusion for 10 minutes. For recordings, the dye was excited with a tungsten-halogen light source (Oriel Instruments, Stratford, CT) through a 514±5 nm band pass filter. The fluoresced light was high-pass filtered at 610 nm and recorded onto a 16×16-element photodiode array (model C4675; Hamamatsu, Japan) through high numerical aperture photographic lenses using the tandem-lens configuration (85 mm, F/1.4 and 105 mm, F/2.0, Nikon, Japan). The optical signals were amplified with a variable gain (1×, 50×, 200×, 1000×), filtered with a variable cutoff low-pass filter, AC coupled with a variable time constant (1.8sec., 2.2sec., 10sec., DC). Membrane potentials were recorded with sufficient voltage (1 mV), temporal (0.3 msec.) and spatial (0.9 mm) resolution to monitor the time course of the action potential simultaneously from 256 sites in a 4.5×4.5 mm area (~350 μm resolution between recording sites). Activation times of LA epicardial surface were recorded
during constant rate pacing at cycle length 400 msec.

**Histological Evaluation:** Atrial tissue was fixed in 10% formalin, embedded in paraffin, cut to 5-μm thickness and stained with Hematoxylin and eosin (H-E) or Masson's trichrome. Samples were examined at 200X magnification in a random order and blinded fashion by two observers (TI, JEF). For each reviewer, all samples were evaluated in one sitting, using the same microscope at the same magnification. The reported histological score is the average of these observations. Variables included nuclear enlargement, cellular hypertrophy, cellular myolysis, interstitial fibrosis, and interstitial inflammation, as previously reported. Each sample was graded on a scale of 1-5 based on the extent of a given observation throughout the sample. In general, a score of 1 signified complete absence of the observed variable; 2 indicated that the observation in question was occasionally present (i.e. occasionally notable nuclear enlargement with mostly normal nuclear size); a score of 3 was given for diffuse abnormalities encompassing approximately half of the cells or tissue, 4 was given if the observation in question was present diffusely throughout the tissue, and a score of 5 indicated near complete presence of the variable under examination. We quantified fibrosis in Masson’s trichrome stained sections with the Image-J software (NIH, Bethesda, MD).

**Western Blot Analysis:** The atrial tissue was snap frozen, homogenized, and dissolved by 0.3M sucrose/10mM sodium phosphate buffer containing protease inhibitor (Roche, Indianapolis, IN) and phosphatase inhibitor (Sigma, St. Louis, MO). Ultracentrifugation
(50KxG for 60 minutes at 4°C) was performed to isolate the membrane fraction. Protein concentrations were determined by the BCA method (Thermo Scientific, Rockford, IL). Proteins were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, blocked with 3% non-fat dry milk, and blotted with anti-Cx40 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Cx43 (Invitrogen, Carlsbad, CA), and anti-GAPDH (Santa Cruz Biotechnology) overnight at 4°C. Membranes were washed with blocking buffer and incubated with secondary antibodies directed against the primary and conjugated with horseradish peroxidase. Bands were detected with the enhanced chemiluminescence assay (Thermo Scientific) and quantified using the Image Quant software package (NIH). Band intensity for the protein under question was normalized to the intensity of GAPDH in each lane.

**Immunohistochemistry:** The atrial tissues were fixed in 10% formalin, embedded in paraffin, sectioned at a thickness of 5-µm, and mounted on gelatin-coated slides. The sections were deparaffinized, placed in citrate buffer, boiled in a microwave oven for 11 minutes, and incubated overnight with anti-Cx40 antibody (1:100 dilution, Santa Cruz Biotechnology) and anti-Cx43 antibodies (1:400 dilution; Invitrogen), and then incubated with Cy3-conjugated donkey anti-goat IgG or FITC-conjugated goat anti-rabbit IgG (1:400 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) before being examined by laser scanning confocal microscopy (×40 oil immersion lens, Airy 1 pinhole). The degree of confocality was kept constant for each experiment to minimize overlap of the Cx43 label.
The signal intensity of connexin proteins in the intercalated disk and lateral membrane was digitized and quantified with the Image-J software (NIH).

**Statistical Analysis:** Investigators performing subjective measures were blinded to animal study group. Blinding was achieved by assigning random study ID numbers to each animal or data element by Lizhu Yang, who kept the assignment information secure until data compilation and statistical analysis.

Statistical analyses were performed as follows: for rhythm analysis, the probability of SR was calculated as the number of SR segments divided by the total number of off-pacing segments per daily ECG recording and assessed by Poisson regression using generalized estimating equations in order to address the animal-level clustering. In optical mapping, histology, immunohistochemistry and western blot analyses, statistical differences were determined by repeated-measures ANOVA (post-hoc Tukey correction), which assumed that outcome was correlated over time for each animal. All statistical tests were conducted at the 0.05 significance level. Statistical analysis using parametric model were performed using SAS9.2 GLM and GENMOD procedures. All data are presented as means±SD.
References


